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THE BIODEGRADATION OF FUELS IN SOILS AND SEDIMENTS: DIFFERENCES AS A FUNCTION OF MINERALOGY

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ABSTRACT

Experiments were carried out to determine the effects of substrate mineralogy on the biodegradability of fuel components. Samples of quartz sand (Fischer Sea Sand) and illite clay (API#35) were spiked with DFM, aged, slurried and inoculated with DFM-acclimated soil microorganisms. Then, the concentrations of fuel components were monitored over time. While there was clear chromatographic and biomarker evidence of TPH biodegradation on the sands, illite samples showed no evidence of biogenic loss of aliphatic components. PAHs, on the other hand, degraded equally well on both substrates, and in both cases, degraded to a much greater extent than did TPH.

INTRODUCTION

Petroleum contaminated soils and sediments are highly complex systems. Soils and sediments are composed of one or many minerals and naturally occurring organic matter, exhibiting varying properties such as surface chemistry, grain size and porosity. Petroleum products are mixtures of hundreds of aliphatic and aromatic organic compounds, the relative proportions of which vary greatly between fuel type and somewhat between batches of the same type. Each fuel component differs in its reactivity, solubility,[1,2] volatility, mineral surface affinity,[3,4] and biodegradability.[5,6] Furthermore, the mode of introduction of the contaminant into the soil or sediment, postdepositional weathering and diverse mobility characteristics can also drastically alter the composition of the bulk contaminant. A careful consideration of these variables is required in order to choose and design intelligent management options for such complex systems. Due to the unique properties of each fuel and each waste site, there is not one correct remedial approach that can be applied to all contaminants or even all spills of the same contaminant. However, little work has been done to examine these component and mineral-specific effects on bioavailability, mobility and degradability of fuel components.

Remediation technology is often developed and demonstrated on soils and sediments composed of pure, simple and relatively inert quartz sand,[7-12] but the bulk of soils and sediments are actually complex mixtures of materials, including many reactive and high surface area components such as clay minerals, organic matter and metal sulfides and/or oxyhydroxides. A key factor in the remediation of the most recalcitrant fractions of hazardous organic waste is the long-term sorption that can occur between organic molecules and clays or other minerals in soils and sediments.[13] Organic-mineral binding mechanisms are poorly understood,[14] and the fundamental chemistry controlling these important issues in natural systems must be determined before it will be possible to develop an intelligent approach to remediation of contaminated sites. We have shown, for instance, that the rate and nature of interaction and weathering of fuels on pure sands and pure clays is fundamentally different, probably because of different mineral/contaminant interactions taking place on the clays and sands.[15-17]

Due to the large differences in sand and clay specific surface areas (*e.g.*, ~0.2 m^2/g for sand, and ~80 m^2/g for illite), fine-grained minerals, even if in small proportions, can comprise the bulk of the surface area of soils. For instance, less than 1% illite by weight in sand will double the specific surface area of the resultant mixture. When soils and sediments are separated by grain size, the bulk of contamination is concentrated on the fine-grained clay particles.[18-22] Thus, even in predominantly coarse materials, contaminant behavior may be controlled by the fines, primarily the clays. These minerals can form physical or chemical interactions with contaminant components which may strongly affect contaminant bioavailability, transport, extractability or degradability. The rate of release of these contaminants to the environment, and thus, the relative risk in each case, may differ as a function of mineralogy.

The purpose of this experiment was to take a first look at the effects of substrate mineralogy on the

biodegradability of fuel components. In order to achieve this, representative "endmember" minerals, a quartz sand and an illite clay, were chosen.

METHODS

The substrates used were single component soils (Fischer Sea Sand and illite clay - API#35[23-25]). Both the sand and illite clay were lightly ground and meshed to the same size class: 25-140 mesh (710-104 μm). The clay samples were not individual mineral grains at this size class, but were clumps or aggregates of grains which adhered together as the result of minor, possibly calcareous, cementation. The sands, however, consisted of individual mineral grains, primarily of quartz (>99.9%). Figure 1 shows Scanning Electron Microscope (SEM) photographs of both the Fischer Sea Sand and illite, at the same magnification. Measurements of the specific surface areas (SSA) of sand and illite at the mesh sizes used, by adsorption of N_2 gas (BET method[26-28]), show that illite has a significantly higher surface area (~78 m^2/g) than does quartz sand (~0.2 m^2/g).[15]

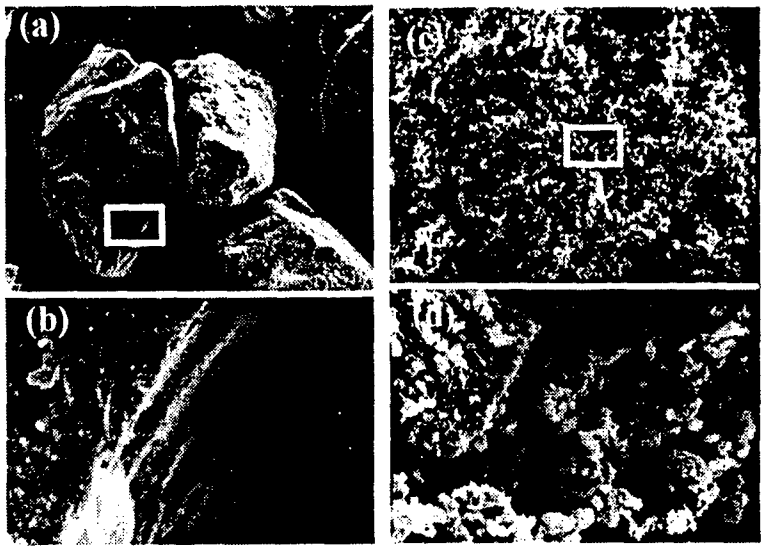

Figure 1. SEM images of Fischer Sea Sand (a and b) and API#35 illite (c and d). Upper images are 200X, lower images are 8X magnifications of the framed portions of the upper images

Sand and clay samples were weighed in 5 g aliquots into vials, and then fuel (diesel fuel marine, DFM, from the Navy Fuel Depot, San Diego) was added to yield concentrations of 0 to ~35 mg DFM/g dry substrate. The samples were placed on a Cole-Parmer Roto-Torque for two weeks. After being used as standards for a fluorescence experiment, samples were allowed to age in vials, which were not air-tight, for two years. No attempt was made to keep the samples abiotic. After two years, samples were combined (all the sands together and all the clays together) and homogenized. Homogeneity of the combined samples was confirmed by bulk fluorescent measurements of subsamples of the materials. Calculated nominal concentrations were about 10 mg DFM/g substrate.

In order to carry out biodegradability experiments, aliquots of these samples were made into slurries. Slurries were prepared by adding one part soil (1 g) to two parts mineral salts solution (2 ml) in 20-ml, sterile vials with foil lids. The slurries were inoculated with a population of DFM-acclimated soil microorganisms. The microorganisms were originally obtained from a jet fuel-contaminated site and subsequently grown on DFM as the sole organic carbon source. Abiotic controls were prepared by adding mercuric chloride to the slurries.[29] Sterility of the controls was confirmed by lack of microbial growth on tryptic soy agar (TSA) plates. The slurry vials were shaken at 250 rpm on a reciprocal shaker under ambient laboratory conditions. Triplicate samples were periodically sacrificed for cell density determination and chemical analyses. Microbial densities were based on the colony forming units (CFUs) of serial dilutions on TSA plates.

Samples were prepared for total petroleum hydrocarbon (TPH) and polynuclear aromatic hydrocarbon (PAH) analyses. Slurries were acidified with 100 μl of concentrated hydrochloric acid and then extracted for three minutes with 16 ml of a 1:1 mixture of methanol and hexane. The hexane extracts were analyzed by gas chromatography with flame ionization detection (GC-FID) for TPH content and by GC-mass spectrometry (GC-MS) for PAHs.

Both forms of GC analyses were carried out on Hewlett Packard 5890 GCs, equipped with 12 m x 0.2 mm i.d. capillary columns with 0.33 μm thickness of cross-linked methyl silicone gum. For GC-FID analysis the oven

temperature started at 35°C for 0.6 min, ramped 10°C/min to 180°C, and then ramped 20°C/min to 230°C. The temperature was held at 230°C for a total run time of 30 min. TPH concentrations were determined by the internal standard method, with tert-butylbenzene added as the internal standard. For GC-MS analysis the starting GC temperature was held at 35°C for 0.6 min and was ramped 20°C/min to 240°C and held there for a total run time of 15 min. The MS was run in selective ion mode, with chosen *m/z* values correlating to the parent ion of those PAHs and other unsaturated cyclic compounds known to be in DFM (Battelle, Northwest Laboratory, pers. comm). Trimethyldibenzothiophene, a component of DFM that is fairly resistant to biodegradation, was treated as a conserved internal biomarker or "built in" internal standard. PAH data are reported as the area of the GC-MS peak(s) normalized to the area of trimethyldibenzothiophene.

It should be pointed out that one potential difficulty of using aged, contaminated materials, is reduced extraction efficiency of analytes due to weathering and time. It is well known that due to sorption processes, analyte recovery decreases with the aging of samples.[15,17,30] This can make mass balance considerations especially difficult. Furthermore, since the production of biosurfactants during slurry treatment may *increase* extractability *over time*, this may further confound interpretation of biodegradation experiments. Thus, it is important to remember that, as an experiment progresses, extractability may change, and that all concentrations reported are *extractable* concentrations, and not necessarily *total* concentrations.

RESULTS AND DISCUSSION

As was stated above, the spiked sand and illite samples were allowed to "weather" in vials for about two years before the experiment was begun. Thus, "Day 0" results, as reported in the following figures, are actually from well-aged materials. Since the vials were not air-tight, and the samples were not sterile, some volatilization, and perhaps degradation, of the DFM components on the substrates occurred even before this experiment began. Different degrees of volatile loss from the sand and illite are apparent in the GC-FID chromatograms in Figure 2. The "Clay - Initial" sample shows much less evidence of volatilization, with taller and more abundant peaks on the left-hand, lighter end of the chromatogram, when compared to the "Sand - Initial" chromatogram. This suggests that the clays better retain the volatile components, inhibiting volatile loss. This also means that the sand and clay samples, though always treated the same, started the biodegradation experiment with different compositions (*e.g.*, the clay had a higher TPH concentration and a larger proportion of volatile and biologically labile components). This will again be apparent in subsequent figures comparing TPH and PAHs in sands and clays.

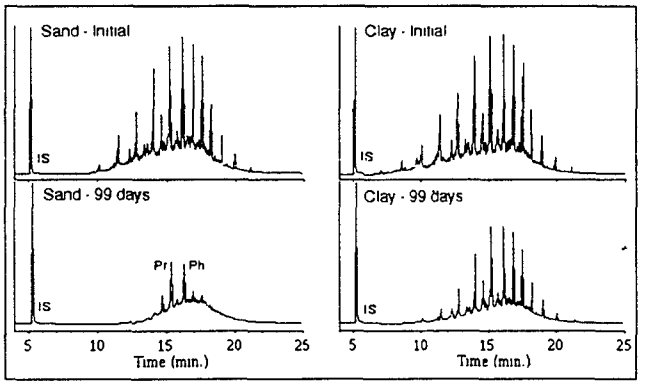

Figure 2. GC-FID chromatograms of sand and clay extracts at Days 0 and 99. Labeled peaks are the internal standard (IS), pristane (Pr) and phytane (Ph).

TPH RESULTS

The bottom portion of Figure 2 shows the chromatograms of the sand and clay extracts after 99 days in the bioslurry. The chromatograms of the sand and clay extracts differ in important ways. First, the sand has significantly fewer and smaller peaks standing above the unresolved complex mixture (UCM), or hump of the chromatogram. The majority of the depleted peaks represent the normal alkanes, which generally disappear rapidly when biodegradation occurs. This suggests that the *n*-alkane portion of the DFM on the sand was effectively biodegraded. The clay sample, on the other hand, still contains high levels of *n*-alkanes, even after 99 days in the slurry. Though some of the lighter peaks on the left side of the chromatogram have decreased, and total concentration has reduced, the chromatogram seems to reflect volatile loss more than a classic pattern of biodegradation. In fact, the clay chromatogram after 99 days looks much like the sand chromatogram did at Day 0.

Percent extractable TPH (Figure 3) drops in both the sands and clays over time. However, the results in illite were much noisier, and the drop was only by about 40%, while the drop in sand was about 50%. When compared to

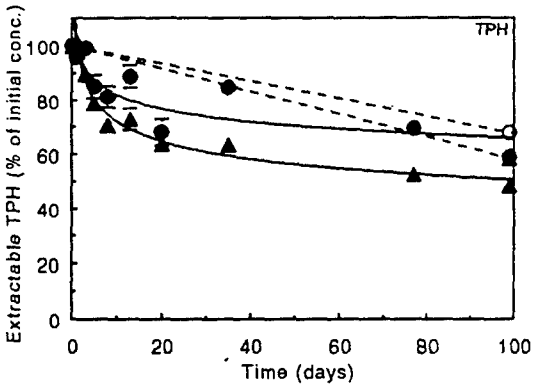

Figure 3. Extractable TPH concentrations as a percentage of initial concentrations vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

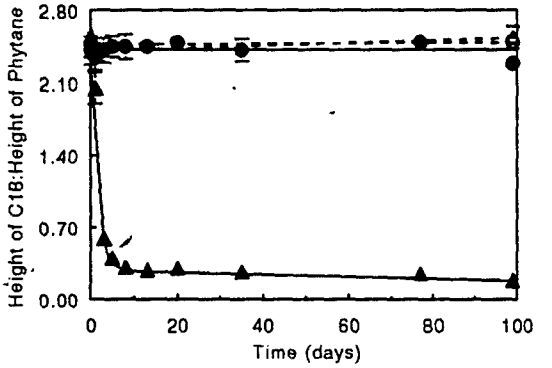

Figure 4. C18:phytane ratios in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

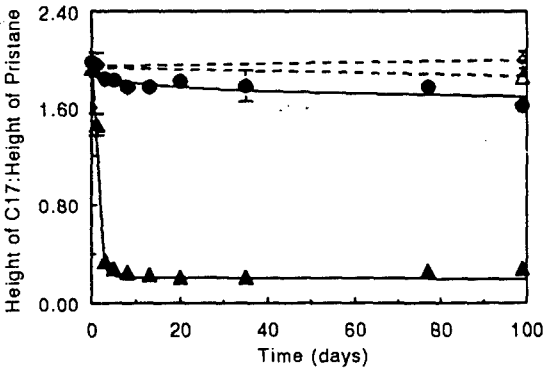

Figure 5. C17:pristane ratios in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

their respective poisoned controls, the biotic samples appear to have lost only 10% additional TPH. Poisoned controls were only measured for Days 0 and 99. Lines connecting the data points are for visual purposes only, and are not meant to provide any mechanistic insight into projected trends between these days. It is not likely that these trends were actually linear.

The apparent lack of biodegradation of the TPH components on the illite when compared to the sand is further bolstered by *n*-C18 to phytane and *n*-C17 to pristane ratios, from the chromatograms, over time. These ratios have traditionally been used to distinguish biodegradation from abiogenic processes such as physical weathering, volatilization and leaching.[31-33] The straight chain alkanes (C17 and C18) are readily biodegradable and have similar volatilities to their respectively mated branched alkanes (pristane and phytane), which are less biodegradable. A drop in the ratios indicates biodegradative loss of the *n*-alkanes. The C18:phytane ratios for the illite samples and both poisoned controls stay flat for the 99 days of the experiment (Figure 4), while within eight days the sand samples drop rapidly from about 2.5 to about 0.3, which is very near the detection limit for C18 above the UCM. The C17:pristane ratios for the clay samples appear to drop very slightly relative to the poisoned controls (Figure 5). The sand samples, on the other hand, drop dramatically in the first 4-5 days. That pristane and phytane are relatively resistant to biodegradation is evidenced by the fact that they comprise two of the only remaining peaks visible above the UCM for the "Sand - 99 day" chromatogram in Figure 2. While not absolutely definitive, the ratios strongly suggest, as did the chromatograms themselves, that biodegradation is either not occurring or is occurring at very low rates in the illite samples, when compared to the sand samples.

Examination of Figures 2-5 would strongly suggest that, based upon the classic indicators of fuel biodegradation, the API#35 illite strongly inhibits bioremediation of fuel components, and that any loss observed appears to be a volatile loss. This is quite a distressing result, since, as discussed above, even in small proportions, clay in a soil or sediment can dominate the surface area, and possibly, contaminant behavior.

MICROBIOLOGICAL RESULTS

Figure 6 shows microbial densities, in log CFUs/ml. Day 0 reflects the microbial densities in the slurries right after being inoculated with the DFM-acclimated organisms. Background levels of microorganisms for the sand and clay were <10 and <1000 CFUs/ml, respectively, suggesting that the materials had very low microbial populations before inoculation. Within a day of inoculation, populations on both substrates rose ten-fold to near 10^8 CFUs/ml. Levels remained vigorous throughout the experiment, dropping slightly near the end, possibly reflecting a depletion of mineral salts and/or production of a toxic byproduct. Of note is the fact that microbial populations were as high or higher in the clay samples than in the sand samples, suggesting

that microbial activity (and thus, utilization of some organic carbon source) was occurring in the clay as well as the sand samples.

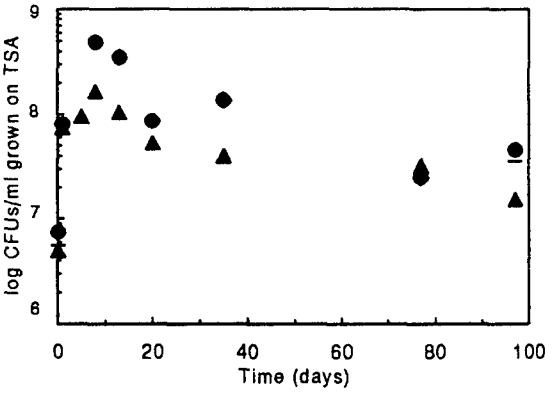

Figure 6. Microbial densities vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands.

Although we find no evidence in the literature,[23-25] there is some evidence that the API#35 illite may contain some natural organic matter. C-H-N measurements of the illite showed 1.6 - 3.5 wt. % C, and 0.13 - 0.28 wt. % N. Treatment with HCl generates fizzing, suggesting that at least some of the C may be in the form of a carbonate cement; but organic vs. inorganic C values have not yet been determined. In previous, related work, fluorescent peaks were sometimes observed in illite-DFM extracts that were not observed in sand-DFM extracts, suggesting some, possibly organic, fluorophore in the illite. However, we have not been able to reproduce those peaks in extracts of illite without DFM. It is possible, however, that some natural organic matter occurs in the illite, which may either provide a carbon source for some of the microbes or may inhibit alkane biodegradation. No C or N was observed in C-H-N measurements of the sand samples.

PAH RESULTS

Because PAHs comprise the most potentially toxic fraction of many petroleum fuels,[34] they have merited much attention in recent years. It has been demonstrated that soils and sediments exposed to fuel contamination often contain PAH degrading organisms,[35] and that under favorable conditions, PAH degradation can occur at a measurable rate.[36,37] The more toxic, multi-ring compounds, however, are often subject to slow or negligible degradation rates due to their chemical complexity[36,37] and tendency to partition to the solid substrate.[14,38]

PAHs comprise about 3% by weight of fresh DFM (Battelle, Northwest Laboratory, pers. comm). Since they are minor components of the fuel, they tend to be buried in the UCM of the GC-FID chromatograms. Figures 7-15 show the concentrations of various PAHs in extracts over the course of the experiment, based upon GC-MS analyses. All PAHs that were detected and measurable in the samples are reported. The simplest and most volatile aromatics, such as naphthalene, were not observed in these samples, probably because they had ample time to volatilize during sample weathering. Those which were observed will be discussed from the simplest to the most complex.

Methylnaphthalene is one of the most volatile PAHs detected in the samples. This is apparent by the nearly complete loss of this component in the sand samples during sample weathering (Figure 7), as seen in the much lower concentrations in the sand than the clay samples at Day 0. On both substrates, however, concentrations rapidly drop to very low levels in the first 10-20 days. It is not possible, however, to determine whether this loss was by biodegradation or volatilization, since the poisoned controls dropped to similarly low levels.

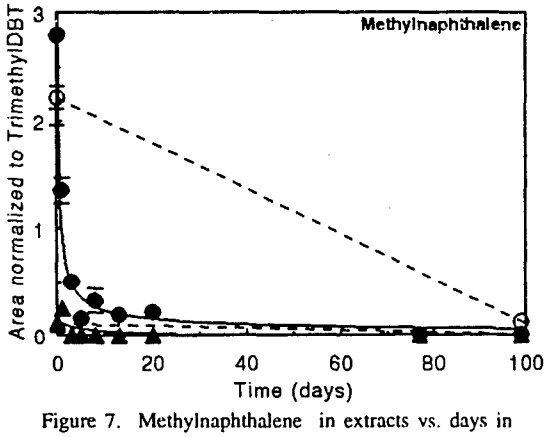

Figure 7. Methylnaphthalene in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

It should be pointed out that having abiotic and biotic samples dropping to the same low level does not necessarily indicate that biodegradation is not occurring in the live samples, but rather that it cannot be separated from the abiogenic processes causing the loss in the killed samples. Furthermore, a lower value in the biotic relative to the abiotic sample does not *prove* biodegradation, since adding the poison may change the slurry characteristics relative to the non-poisoned sample. It does, however, provide a strong suggestion of biodegradation. Only the detection of degradation products or the mineralization of labeled

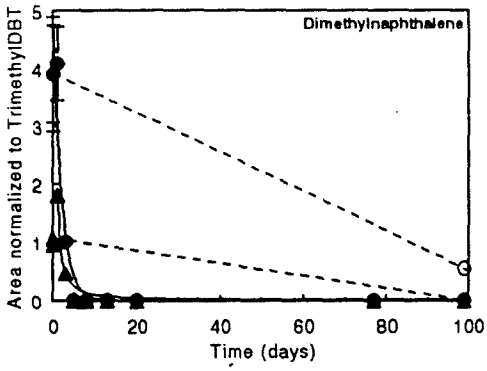

Figure 8. Dimethylnaphthalene in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

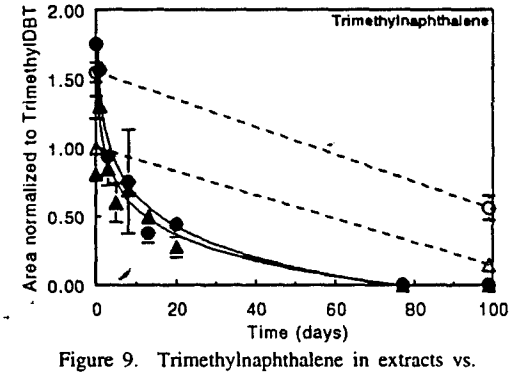

Figure 9. Trimethylnaphthalene in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

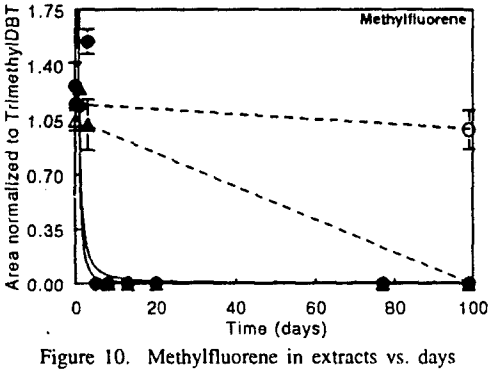

Figure 10. Methylfluorene in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

compounds would be definitive evidence of biodegradation.

Dimethylnaphthalene (Figure 8) is somewhat less volatile than methylnaphthalene, as reflected in the higher levels in the sand samples relative to methylnaphthalene at Day 0. Loss of this component is very rapid on both substrates, dropping to undetectable levels in the first five days. Although the sand poisoned controls drop to as low a level as the live sand samples, the clay poisoned controls do not drop as low. This suggests two things: that the illite inhibits volatilization of the dimethylnaphthalene (reflected in the clay control not dropping as low as did the sand control); and that some biodegradation of this PAH may be occurring in the live illite samples (reflected by the live samples having lower values than the killed samples). Trimethylnaphthalene tells a similar story (Figure 9), but the slight offset between the live and killed sand samples suggests that some biodegradation, rather than purely volatilization, is occurring on the sand.

Methylfluorene (Figure 10) drops off precipitously in the first 4-5 days in both the sand and clay samples. However, as in dimethylnaphthalene, the abiotic and biotic sand samples at Day 99 are indistinguishable. In the clay samples, however, the offset between killed and live samples is substantial, with killed samples showing very little reduction, and live samples dropping below the detection limit. Just as for dimethylnaphthalene, this suggests both that the clay can inhibit volatilization, and that extensive biodegradation may be occurring in the live clay samples.

Methylanthracene and methylphenanthrene (Figure 11) are indistinguishable by our current analytical method, so they are reported together. Both the sand and clay live samples drop to the detection limit in about 20 days, about at the same rate. However, both sand and clay killed samples show only negligible drops over the 99 days of the experiment. These significant offsets between the live and killed samples strongly suggest that biodegradation of these compounds is occurring on both substrates.

Similarly, dimethylanthracene and dimethylphenanthrene (Figure 12, again, reported together) show drops to similar levels on both live substrates. In both abiotic sets of controls, the drop is negligible. These offsets between the live and poisoned samples suggest that biodegradation of these compounds is occurring on both substrates. These compounds are somewhat resistant to biodegradation; thus, in neither of the substrates does the level drop to the detection limit. Trimethylanthracene and trimethylphenanthrene (Figure 13, reported together) are at levels near the detection limit of our method, and so, no clear statement can be made about whether these PAHs are decreasing or staying constant for any of the samples.

The sum of the measured PAHs provides further insight into the PAH behavior on these substrates. The total PAH (TPAH) of the live samples of both sand and clay (Figure 14) drop to about the same level, possibly suggesting that this is a level at which the organisms in this mixture cease to respond to the PAHs, or that conditions in the sample vials are no longer conducive to further degradation. On the other hand, this correlation may be fortuitous, and this requires further investigation. Killed illite samples, which started at a higher level and with a higher

proportion of volatile components than did the sand samples, show a slightly greater net reduction than do the killed sand samples, but both result in TPAH that is clearly above that in the live samples. When plotted as percent of initial extractable TPAH, live sand samples drop by about 80%, while clay samples drop about 90% (Figure 15). It should be noted again that clays started out with a higher concentration of volatile and biologically labile components, which may explain some of this offset, since they both level off at the same final concentrations (Figure 14). The percent lost in both poisoned controls is only about 30%, suggesting that biodegradation may account for at least 50-60% of the TPAH loss in the live sand and clay samples, all other things being equal.

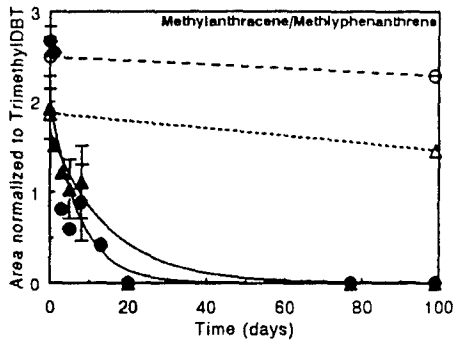

Figure 11. Methylanthracene/methylphenanthrene in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

CONCLUSIONS

In spite of the dire observations of minimal biodegradation of TPH and alkanes on the illite samples by classical analysis, there is strong evidence that PAHs degrade as well on the illite samples as on the sand samples. In fact, based upon percent loss from original concentrations, PAHs show a much more extensive degradation (80-90%, Figure 15), than does the TPH (30-40%, Figure 3) on both substrates. Since PAHs are often the contaminants of greater concern because of potential toxic effects and regulatory scrutiny, this is an exciting result. It is also an indication that simple measures of degradation may not tell the complete story in complex systems. Most experiments have been carried out on simple substrates, often with single contaminants; however, real systems are actually quite complex and merit careful attention.

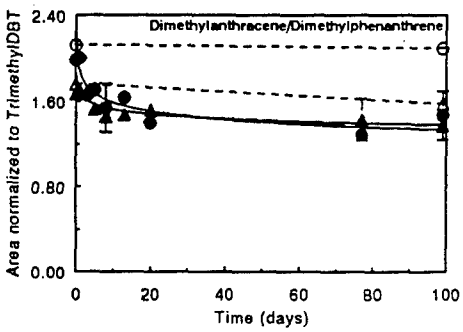

Figure 12. Dimethylanthracene/dimethylphenanthrene in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

These results show clear offsets in the biodegradative potential of DFM components on sand and illite, but several issues remain to be resolved. Illite only represents one of many clay mineralogies. It was chosen for our experiments because it is the most abundant clay in San Diego area sediments,[39] which are being used in several experiments here; but other clays, such as montmorillonites and kaolinites, need to be examined, as well as metal sulfides and oxyhydroxides. Labeled compounds need to be used to unequivocally demonstrate biodegradation, and to perform complete mass balances. The role of natural organic matter vs. mineralogy needs to be clarified, and whole soils and sediments must be examined more carefully.

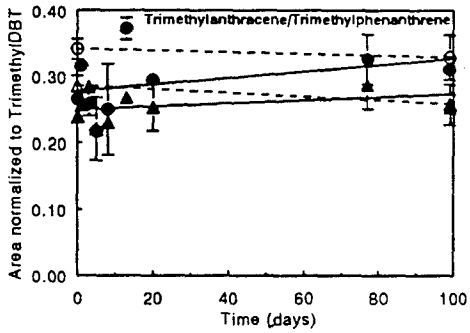

Figure 13. Trimethylanthracene/trimethylphenanthrene in extracts vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

In a thorough survey of soil-microbial interactions, Stotzky[40] notes that the understanding of clay-organic-microbial interactions is not very extensive, and quite equivocal. When organics are sorbed to clays, either reduced or enhanced bioavailability can result, depending on mineralogy, organic composition and experimental conditions. Sometimes clays can act as concentrators, and thus, sources of organic nutrients, while at other times, they can inhibit mineralization. What controls these processes remains unclear, and practically unexamined for petroleum components. Stotzky states:

> "...the paucity of data on the adsorption and binding of hydrophobic substrates on clay minerals and on the subsequent microbial utilization of these substrates indicate that more studies with hydrophobic compounds and clay minerals - both "clean" and "dirty" - are warranted."

In spite of the passage of eight years since this statement was made, this still holds true.

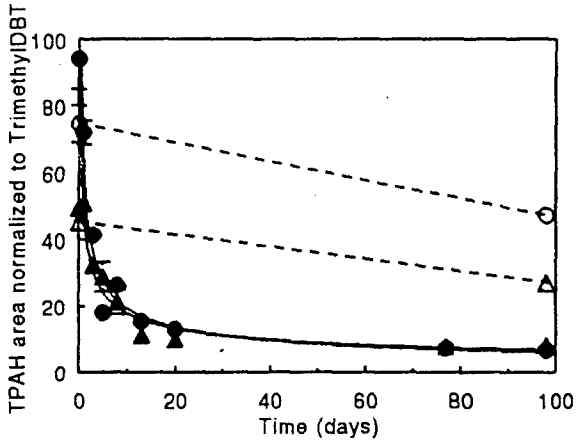

Figure 14. Sum of all PAHs measured vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

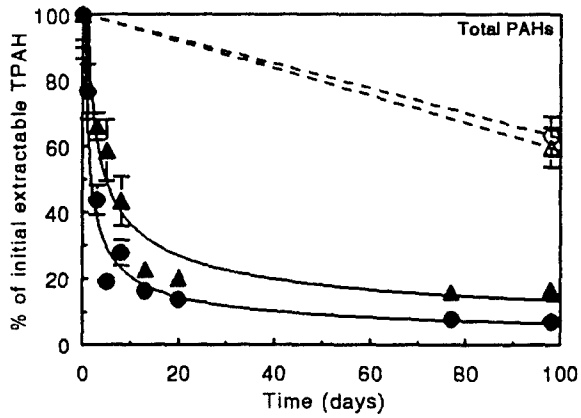

Figure 15. Sum of all PAHs measured, as a function of percent of initial extractable TPAH vs. days in slurry. Filled circles are inoculated clays, filled triangles are inoculated sands, open symbols are the corresponding poisoned controls.

There are several possible ways to explain the observed differences of the alkane and aromatic behavior on sands and clays. These include differential water solubilities, surface affinities, or molecular sizes (and thus the ability to "hide" in mineral pores). Clearly, aromatics are generally more water soluble than are alkanes, and may be less subject to partitioning on the clay surfaces, and thus may be more bioavailable. However, since even the least soluble PAHs measured seem to biodegrade on the illite samples, the issue is probably more complex than simply water solubility. These issues are outside the scope of this preliminary paper. But, an understanding of mineral-organic interactions may be critical to the success or failure of bioremediation attempts in soils and sediments, so they merit further study.

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